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Army Biological Defense Research Lab ltr, 8 Dec 1971

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON *

Sixth Quarterly Report of Progress

on

Research Project Number 4B04-14-004
Order Number FDO-5013

October 1 - December 31, 1961

Conducted by

Milk and Food Research, SEC

for the

U. S. Army Chemical Corps Biological Laboratories
Fort Detrick, Maryland

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio
January 1962

* "In conducting the research reported herein, the investigator(s) adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care' of the Institute of Laboratory Animal Resources, NAS-NRC"

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ABSTRACT

Serological studies on the immunogenic properties of biphenyl bridge conjugates of paralytic shellfish poison and protein (PSP-azo-biphenyl-azo-ovalbumin) indicate that antibodies produced by rabbits were directed against the protein portion of the molecule and that the PSP moiety did not possess haptenic properties.

A method of coupling PSP to protein in an acid media through the intermediate action of formaldehyde is also described. Evidence is presented which suggests that the PSP is only slightly altered in this synthesis.

CONTENTS

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

I. Introduction

In attempting to determine the haptenic properties of paralytic shellfish poison (PSP) the primary investigative format has consisted of joining derivatives of the poison to the antigenic proteins followed by a determination of the immunological and serological properties of the resulting product. Prior to this report these studies have been substantially limited to derivatives of PSP produced through the reaction of nitrous acid and coupled either to ovalbumin or bovine gamma II globulin. Evidence collected on the immunological properties of such conjugates has revealed that they are capable of eliciting antibody production in rabbits which may be demonstrated by precipitin reactions, inhibition tests, skin tests, and complement-fixation tests. Haptenic properties of the non-protein portion of the conjugates, diazotized PSP, were also demonstrated by means of inhibition or precipitin reactions, complement fixation tests and skin tests. However, none of the several serological techniques employed provided any conclusive evidence that antibodies to unaltered PSP were produced. Accordingly, other means of conjugating PSP derivatives to proteins have been investigated. Studies on the reaction between PSP and aromatic diazonium salts gave rise to a method of coupling through a tetrazotized benzidine intermediate described in the previous quarterly report. This report includes an immunological evaluation of conjugates prepared in this manner and a method for coupling PSP to proteins under acidic conditions.

II. Experimental

Immunological studies related to determining the antigenicity of PSP-azo-biphenyl-azo-ovalbumin and biphenyl-azo-albumin.

PSP-azo-biphenyl-azo-ovalbumin and biphenyl-azo-ovalbumin were prepared as described in the Fifth Quarterly Report of Progress (July-September, 1961). The antigens were maintained in the frozen state (-10°C.) and used to immunize two series of four rabbits each, according to the schedule shown in Table 1. The individual sera collected from rabbits of both series were titrated against the two antigens to determine the relative concentrations of precipitating antibodies present. The results of these primary titrations are given in Tables 2 and 3 and reveal that relatively high titered sera were obtained. To differentiate among the possible antibodies present in these sera, a series of Ouchterlony gel-diffusion plates were prepared in which serum at a 1:5 dilution was allowed to react with the following antigens: PSP-azo-biphenyl-azo-ovalbumin; biphenyl-azo-ovalbumin; ovalbumin; biphenyl amine; PSP; PSP-azo-biphenyl; PSP-azo-biphenyl-azo-bovine serum albumin; and biphenyl-azo-bovine serum albumin. The following concentrations of each antigen were employed: undiluted, 1:50, 1:100, 1:150, 1:200, and 1:250. A single, continuous band of precipitate was formed with both types of sera to PSP-azo-biphenyl-azo-ovalbumin, biphenyl-azo-ovalbumin, and ovalbumin. No bands of precipitate were formed to the remaining

five antigens. These results indicated that antibody had been formed only to ovalbumin and the results depicted in Table 1 and 2 reflect the reaction of ovalbumin and its antibody. In view of the lack of demonstrable precipitating antibodies to PSP or its biphenyl derivatives, tests were performed to determine the presence of antibodies capable of inhibiting the reactions of the sera to their homologous antigens. Employing the inhibition procedure described in previous reports, no inhibition of reaction was noted by the addition to the systems of PSP, biphenyl PSP, biphenyl amine, PSP-azo-biphenyl-azo-bovine serum albumin (BSA) and biphenyl-azo-BSA. The results obtained with serum 94 are characteristic of those observed and are presented in Table 4.

To determine the presence of passively transferable protective antibodies in the sera, a series of protection tests were performed with mice, employing the procedure outlined in the Fourth Quarterly Report of Progress. The mice were challenged with 0.5 μg. of PSP contained in 1.0 ml. of sterile, distilled water. All mice in both the protected and unprotected groups which received toxin died within 4.0 to 5.5 minutes, indicating the absence of protective antibodies in the sera.

A method for conjugating PSP with proteins under acidic conditions.

In all previous preparations for PSP conjugated proteins it has been necessary to subject the reaction mixtures to neutral or weakly alkaline conditions for varying lengths of time. Although no direct

evidence has been developed to show the extent of structural change undergone by PSP, the immunological investigations and toxicity studies indicate that substantial changes do occur. In view of these observations it was determined that future preparations of PSP-protein conjugates should meet the following two prerequisites: (a) PSP should at no time prior to injection be subjected to a pH higher than 4.5 and (b) the chemical reaction used in the coupling should be of the simplest possible nature. In an effort to satisfy these requirements investigations into the feasibility of coupling PSP to proteins by the intermediate action of formaldehyde show promise. By this method Fraenkel-Conrat and Olcott (J.A.C.S. <u>70</u>:2673-84. 1948) were able to couple simple primary amides and substitute guanidines to bovine serum albumin at an optimum pH of 4.2. These workers postulated that the coupling is effected by the introduction of a methylene bridge between free primary amine groups on the protein and the primary amide or guanido nitrogen of the substance being coupled.

$$\text{Protein} - NH_2 + HCHO + \begin{matrix} H_2N-\overset{\overset{\displaystyle O}{\|}}{C}-R \\ \text{(or)} \\ H_2N-C\begin{matrix} \nearrow\!\!\!\!\nearrow NH \\ \searrow NHR \end{matrix} \end{matrix} \dashrightarrow \begin{matrix} \text{Protein} - NH - CH_2 - NH - \overset{\overset{\displaystyle O}{\|}}{C} - R \\ \text{(or)} \\ \text{Protein} - NH - CH_2 - NH - \overset{\overset{\displaystyle NH}{\|}}{C} - NHR \end{matrix}$$

Since it is possible to obtain guanidine and/or β-guanidopropionic acid as degradation products of PSP, it is not unreasonable to expect PSP to be reactive in such a coupling scheme. It has been found in

toxicity studies on dialysates of reaction mixtures containing PSP, protein and formaldehyde that PSP does in fact couple to bovine serum albumin (BSA) under these conditions. BSA was chosen as the protein in these experiments, since it has a high primary amine content (65-70 groups per molecule), and the primary amine has been postulated to be the reactive protein group in this procedure. The general coupling procedure used to date consists of dissolving 50 mg BSA in 4.0 ml 0.1 M acetate buffer at pH 4.2, adding 0.5 ml of a 5% solution of formaldehyde and making the solution up to 5.0 ml by addition of 0.5 ml of a PSP solution containing 1.92 mg/ml. This reaction mixture is then allowed to set at 25°C. for 72 hours. The final reaction mixture contains 0.5% formaldehyde. Table 5 shows the effect of formaldehyde concentration and time of reaction on the extent of coupling. It is evident from Table 5 that a reaction time of 72 hrs. with a formaldehyde concentration of 0.5% produces entirely satisfactory results. Increasing the reaction time serves to cause clouding of the protein solution without significantly increasing the amount of PSP coupled. The poison may be quantitatively coupled by increasing the formaldehyde concentration to 1.9%; however, such a high concentration of formaldehyde causes the protein to form a hard gell, making it undesirable for use in immunological work.

Toxicity data show that the coupling of PSP to BSA in the presence of formaldehyde is to some extent reversible. There has been no attempt to recover all the PSP from a coupling preparation, and such an attempt

may not be feasible at this stage, since it has been found that formaldehyde in 0.5% concentration causes a 48% loss in the toxicity of PSP by an unknown mechanism. The quantitative recovery of PSP will be attempted when more is known about this detoxification. For purposes of determing the extent of coupling and reversibility, allowance was made for formaldehyde detoxification by the use of selected control solutions. The following is a description of a typical preparation of PSP conjugated BSA and the manner in which analytical data was obtained.

The reaction mixture and appropriate control solution were made up as described in Table 6.

The solutions were made up to 5.00 ml with water and allowed to react 72 hrs. at 25°C. At the end of this time the solutions were washed into cellophane dialysis bags with five milliliter aliquots of water. The dialysis bags were placed in 50 ml screw cap tubes and covered with 10 ml of water each. These solutions were then dialyzed on the shaker for 3.5 hours at 25°C. These dialysis conditions had previously been established to be sufficient for the attainment of equilibration of PSP. The toxicity of the reaction mixture dialysate was 6.12 μg PSP/ml and that of the control dialysate was 33.5 μg PSP/ml. Comparison of these toxicities indicates that 82% of the available PSP had been coupled to BSA. The reaction and control solutions still contained in the cellophane bags were again dialyzed under identical conditions except that the time was increased to one week. Assay of these second dialysates was expected to reveal that they were just

half as toxic as the first dialysates. The expected result was obtained in the case of the control; however, in the case of the reaction mixture the second dialysate was more toxic than the first, thus indicating that an equilibrium was involved, and that removal of free poison from the reaction mixture by dialysis caused a reversal of the coupling reaction. The ratio of the toxicity of the second dialysate to that of the first was 1.45 for the reaction mixture and 0.44 for the control. The expected ratio was 0.50. Subsequent experiments of the same type revealed the same effect, and the results are shown in Table 7. The data in Table 7 show that for the control solutions the ratio of the toxicity of the second dialysate to that of the first was 0.50 ± 0.06 in every case as anticipated. However, in the cases of all the reaction mixtures, this ratio was significantly higher. These findings suggest that PSP enters into a reversible reaction with formaldehyde and protein.

A different experimental approach afforded further evidence that an equilibrium is involved in the coupling reaction. The cellophane dialysis bags containing the reaction mixture and control solution were dialyzed against 500 ml 0.001M acetic acid at pH 4 for six hours. The acetic acid was renewed after three hours dialysis, and bioassay of the dialysate at the end of six hours indicated no toxicity. One ml. of the contents of each of the cellophane dialysis bags was injected into each of two mice immediately following dialysis. None of the four mice appeared to be affected. On the basis of the known amount of poison

added to the test solutions and on the amount of poison found in the dialysates, it can be reliably calculated that the mice injected with one milliliter of the dialyzed reaction mixture received 70-72 µg. each. Apparently PSP is non-toxic when conjugated to protein. In order to establish whether an equilibrium is involved in this conjugation, the dialyzed solutions were allowed to set overnight at 25°C. and were again checked for toxicity as before. Sufficient PSP had become uncoupled overnight to cause death times of 2' 03" and 4' 17" in the mice injected with the reaction mixture containing PSP, HCHO and BSA. The control solution was non-toxic as before. This is further evidence that an equilibrium reaction is involved in the coupling.

III. PROJECTED RESEARCH FOR THIRD QUARTER FY 1962

Research to be undertaken during Third Quarter FY 1962 will include (1) immunological and serological studies on the conjugates of PSP and protein coupled under acidic conditions as described herein, (2) development of more rigorous evidence concerning the physical and chemical nature of the protein poison conjugate and (3) further study on the nature of the chemical reaction between PSP and the various agents used in producing derivatives for purpose of coupling.

IV. PREPARATION OF PURIFIED SHELLFISH POISON

In accordance with the extended inter-agency agreement for FY 1962 a contract with the State of Alaska Department of Health and Welfare has

been completed and signed for the collection of toxic clam siphons. It is expected collection will begin as soon as weather and tidal conditions permit. The modification of the Food Chemistry laboratories necessary for the isolation and purification of PSP from clam siphons has also been completed.

Table 1.

Immunization schedule for rabbits receiving intravenous injections of PSP-azo-biphenyl-azo-ovalbumin and biphenyl-azo-ovalbumin

Day	Dose
1	1.0 ml.
3	1.0 ml.
5	1.0 ml.
7	1.0 ml.
9	1.0 ml.
11	1.0 ml.
19	Bled

Table 2.

Precipitin reaction of anti-PSP-azo-biphenyl-azo-ovalbumin sera to various antigens

Serum diluted 1:5 0.4 ml. per tube	Antigen	No. of serum	Dilutions of Antigens 0.4 ml. per tube 1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Anti-PSP-azo-biphenyl-azo-ovalbumin	PSP-azo-biphenyl-azo-ovalbumin	93	-	+	+	2+	3+	4+	3+	+	-	-
		94	4+	4+	4+	4+	4+	3+	2+	+	-	-
		95	-	-	+	±	±	+	+	-	-	-
		98	-	-	-	-	+	+	+	+	-	-
Anti-PSP-azo-biphenyl-azo-ovalbumin	Biphenyl-azo-ovalbumin	93	-	-	+	+	2+	3+	3+	2+	-	-
		94	+	2+	3+	4+	4+	3+	2+	+	-	-
		95	-	-	-	-	+	+	+	±	-	-
		98	-	-	-	-	-	+	+	-	-	-
Anti-PSP-azo-biphenyl-azo-ovalbumin	Ovalbumin	93	-	-	-	2+	3+	4+	2+	+	-	-
		94	-	+	2+	3+	4+	4+	2+	+	±	-
		95	-	-	-	-	-	-	+	±	±	±
		98	-	-	-	-	-	-	+	+	-	-

Table 3.

Precipitin reaction of anti-biphenyl-azo-ovalbumin sera to various antigens

Serum diluted 1:5 0.4 ml. per tube	Antigen	No. of serum	Dilutions of Antigens 0.4 ml. per tube									
			1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Anti-biphenyl-azo-ovalbumin	Biphenyl-azo-ovalbumin	81	-	-	-	-	±	+	+	±	-	-
		86	-	-	±	+	+	2+	2+	±	-	-
		87	±	+	+	2+	3+	3+	3+	+	-	-
		88	-	-	-	-	-	±	+	±	±	-
Anti-biphenyl-azo-ovalbumin	PSP-azo-biphenyl-azo-ovalbumin	81	-	-	±	+	±	+	-	-	-	-
		86	+	+	+	2+	3+	2+	+	-	-	-
		87	4+	4+	3+	3+	2+	2+	2+	+	-	-
		88	-	-	-	-	-	-	-	-	-	-
Anti-biphenyl-azo-ovalbumin	Ovalbumin	81	-	-	-	-	-	-	-	-	-	-
		86	-	-	-	-	±	+	+	+	±	-
		87	-	-	±	±	+	2+	2+	+	±	-
		88	-	-	-	-	-	-	-	-	±	-

Table 4.

Effect of various compounds upon the reaction of

PSP-azo-biphenyl-azo-ovalbumin and its homologous antiserum

No. of Serum	Dilution of Serum 0.2 ml. per tube	Antigen 0.4 ml. per tube	Saline 0.2 ml. per tube	Micrograms of inhibiter per tube 0.2 ml. per tube								
				100	50	25	12.5	6.25	3.125	1.562	0.781	0.390
94	Anti-PSP-azo-biphenyl-azo-ovalbumin 1:20 dil.	PSP-azo-biphenyl-azo-ovalbumin 1:256 dil.	+	+	+	+	+	+	BSA biphenyl +	+	+	+
94	"	"	+	+	+	+	+	+	PSP-azo-biphenyl-azo BSA +	+	+	+
94	"	"	+	+	+	+	+	+	Biphenyl amine +	+	+	+
94	"	"	+	+	+	+	+	+	PSP biphenyl +	+	+	+
94	"	"	+	+	+	+	+	+	PSP +	+	+	+

Table 5.

Preparation No.	% Formaldehyde	Time of Rx	% Coupling
I	0.10	48 hrs.	23
II	0.50	48 hrs.	52
III	0.50	72 hrs.	82

Table 6.

Reaction Mixture	Control Solution
4.00 ml. acetate buffer (0.1 M, pH 4.25)	4.00 ml. acetate buffer (0.1 M, pH 4.25)
0.050 ml. PSP solution (19.2 mg/ml)	0.050 ml. PSP solution (19.2 mg/ml)
0.50 ml. 5% formaldehyde	0.50 ml. 5% formaldehyde
50 mg. BSA (bovine serum albumin)	No Protein

Table 7.

Solution	Date of Preparation	Length of Dialysis First	Second	Toxicity of 2nd / Toxicity of 1st
Reaction Mixture	12-13-61	3 hr.	72 hr.	0.75
Containing	12-18-61	3.5 hr.	1 wk.	1.45
PSP+HCHO+BSA	12-22-61	24 hr.	24 hr.	0.76
Control	12-13-61	3 hr.	72 hr.	0.54
Containing	12-18-61	3.5 hr.	1 wk.	0.44
PSP+HCHO	12-22-61	24 hr.	24 hr.	0.49